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A micro-method of protein analysis.

by H. Mattenheimer.

The determination of protein by means of Weichselbaum's (1) biuret method is too inexact when dealing with small quantities of protein (less than 200 γ /ml initial reactant), owing to the low sensitivity of the color reaction. Although we have used this method for protein analysis of enzymatic preparations from amoebae in a simple micro-modification (2) (initial reactant 200 μl with at least 11 γ protein), we nevertheless have searched for a more sensitive colorimetric method. Nielsen (3) recently reported on a method in which the Cu bound by protein in the biuret reaction is analyzed colorimetrically with sodium-diethyl-dithiocarbamate (DDTC). The method becomes about 50 times more sensitive thereby.

The present paper describes a modification of this method which permits protein analysis of very small volumes.

Results and discussion.

Colorimetric determination of protein via Cu bound in the biuret reaction dates back to Shiff (4). He used $Cu_3(PO_4)_2$ as Cu donor, but his method is relatively complicated. Nielsen (3) has modified the method and found advantages in the use of $CuSO_4$, an opinion that we cannot share on the basis of our own research. The amount of colloidally dissolved excess Cu is considerably increased with $CuSO_4$. Nielsen removes the colloidally dissolved Cu by filtration through a G_5 frit. We have established, however, (exp. 3) that filtration holds back not only colloidal Cu but also biuret complex (13%). We do not know whether Nielsen measured biuret simultaneously and noticed the loss. He introduced an empirical frit factor fluctuating from frit to frit. We established that reproducible values cannot be obtained even by using the same frit with thorough cleansing between two filtrations. Moreover, we wanted to dispense with filtration, since we planned to use the analysis as an ultramicro-method for small volumes of protein solutions, e.g. from amoebae. We established that $Cu_3(PO_4)_2$ as Cu donor causes much less Cu to go into colloidal solution (exp. 3; table, column Cu/V). The loss of biuret complex during filtration amounts to only 3% in comparison to 13% in the use of $CuSO_4$. It may be assumed that the greater loss of biuret complex in the use of $CuSO_4$ is attributable to the greater amount of colloidal Cu, which plugs up the frit and holds back the Cu-protein complex. The results are compiled in the following table.

Comparison of $CuSO_4$ and $Cu_3(PO_4)_2$ as Cu donor in biuret and Cu analyses before (B) and after (A) filtration.

Cu donator	Biuret (E_{540})		%	Cu (E_{440})		%
	B	A	loss	B	A	loss
$CuSO_4$	0.242	0.210	13	1.240	0.940	24
$Cu_3(PO_4)_2$	0.242	0.236	3	1.080	0.970	10

The small amount of colloidally dissolved Cu developing by the use of $Cu_3(PO_4)_2$ is easily eliminated by the blank value. In the large number of calibration curves obtained by us, the lines invariably ran through the zero point of the coordinate system after the subtraction of the blank value. Colloidally dissolved Cu cannot be removed completely with a G5 frit upon utilization of either $CuSO_4$ or $Cu_3(PO_4)_2$.

Before resorting to $Cu_3(PO_4)_2$, we worked with a cation exchanger charged with Cu. We obtained minimal formation of colloidal Cu here, but the equilibrium with protein set in only after agitation for several hours, making the method unfeasible for serial analysis. The opposite process of binding Cu subsequently to a cation exchanger is also possible, but unsuited for microanalysis. Attempts were also made to work with dry, powdered $Cu_3(PO_4)_2$, but the reaction was too slow and we recommend the use of freshly precipitated, well-washed, moist $Cu_3(PO_4)_2$. Protected against drying, the preparation may be used for several days.

A fairly strong opalescence was noted upon determination of Cu with diethyl-dithiocarbamate; our calibration curve coincided with that of Nielsen, however. The turbidity may be removed by centrifugation, but not without a loss in color intensity amounting to 70-80%. The opalescence was strongest in connection with the Cu calibration curve, weakest in the determination of serum protein, since various proteins apparently act as protective colloids for the DDTC-Cu complex. We therefore added 0.3% gum arabic (solution C, exp. 2) to the phosphate solution used in the Cu determination and obtained completely clear solutions. This addition is indispensable in the protein analysis of amoebae. The Cu calibration curve depicted in Fig. 1 has a somewhat lower gradient than the curve published by Nielsen.

Our tests confirm a fact known already in connection with the biuret method, namely that a special calibration curve must be established for each protein. For the theory of Cu linkage to protein, see Gurd et al. (5). A curve presented by Shiff (4) indicates that 1 g of serum protein binds 0.145 g of Cu^{2+}. The same value is found in our serum calibration curve (Fig. 2) in conjunction with the Cu calibration curve (Fig. 1).

During the protein analysis of chick serum, in which we initially worked without precipitation of protein, we noted that the calibration curves experienced a progressive rise. Precipitated protein of the same sera produced a linear progression. Since chicks have a blood sugar

content of about 200 mg%, we ascribed the curved progression to glucose and indeed found a progressive rise in a glucose dilution series (Fig.3). Thus a serum with an average content of 100 mg% glucose yields a value based on Cu linkage to glucose corresponding to about 0.4 g% protein, unless the protein is first precipitated out. It is advantageous, therefore, to conduct the analyses with precipitated protein.

After we had established that the use of $Cu_3(PO_4)_2$ as Cu donator makes filtration unnecessary, it was easy to develop the ultramicro-analysis described in exp. 2. The protein calibration curves (Fig. 2) and the glucose curve (Fig. 3) were obtained with the micro-modification. Macro-control preparations invariably yielded identical results.
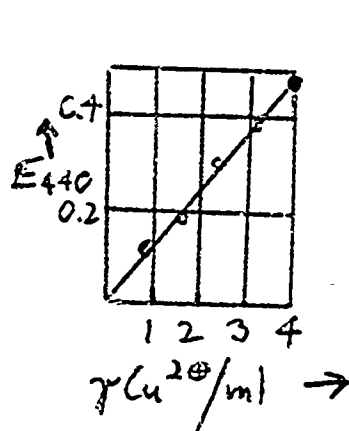
Fig. 1.
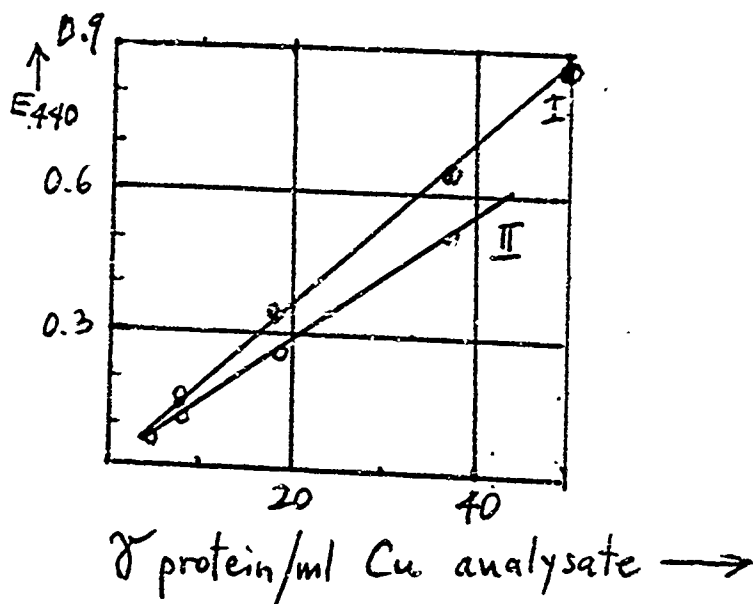Cu calibration curve;
see exp. 1.

Fig. 2. Protein calibration curves.
I. Serum (two test series; ◑ coinciding values). II. Casein, coincides with the curve for amoeba protein.
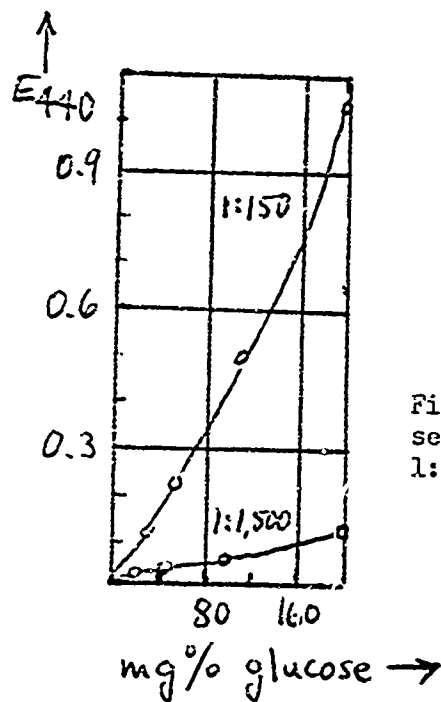
Fig. 3. Cu linkage to glucose;
see exp. 5. Dilutions 1:150 and
1:1,500.

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